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Development of a PCR method to detect

HLA-B27 in ankylosing spondylitis

Ylva Nätterkvist

Supervisor: Greg Bryne

Dublin Institute of Technology, Dublin Ireland.
The aim of the project was to develop a PCR method to detect HLA-B27 at the Immunology Department of St. James hospital in Dublin. The HLA-B27 gene is common among patients with ankylosing spondylitis (AS). Ninety percent of patients with AS have the HLA-B27 gene and it is therefore counted as a risk factor and could be used as part of the diagnosis.

Twenty-two frozen blood samples from patients with AS or suspected AS were donated from the rheumatology department at St. James hospital. PCR is a well known and common technique, many hospital laboratories have a PCR machine and therefore PCR is a good choice for detection of the HLA-B27 gene. A multiplex PCR was developed where a PCR control, primers to the β-globin gene, was used in the same tube as the HLA-B27 primers, to secure that the PCR worked in every tube. Finally a blind test was performed to test the specificity of the PCR.

The result shows that the specificity was 100%. Of all patient samples, sixteen was HLA-B27 positive and six were HLA-B27 negative. In addition, optimal conditions for the PCR and the way to extract DNA from frozen blood were successfully established. For future diagnosis, the described PCR can be used to detect the HLA-B27 gene in patients and it can be considered as a start for further development of a real-time PCR for detection of the HLA-B27.

Keywords: HLA, Multiplex PCR, DNA extraction, hot-start Taq, Spondyloarthropathies.
INTRODUCTION

Ankylosing spondylitis (AS) is a rheumatic disease that belongs to the group of spondyloarthopathies (SpA) diseases. AS is also known as Bechterew's disease. Rheumatic diseases are a common denomination for autoimmune diseases that affects the joints in the body. The most well-known rheumatic disease is probably rheumatoid arthritis. Normally rheumatic diseases affect older peoples. AS is a type of SpA that often starts in young adulthood, between 15-30 years of age, but the diagnosis might be determined long after the symptoms of the disease begins. Today the exact cause of AS is not known but the gene for human leukocyte antigen (HLA)-B27 is present in 90% of all patients with AS and 50-75% of patients with other SpA diseases and therefore it is considered as a contributing factor.

The first symptom for AS is morning stiffness and pain in the lower regions of the back. Initially the pain occurs only in the morning but will be continuous through the day after a few months. The morning stiffness will only be present for a few hours in the morning and can be reduced after exercise. The back pain is usually due to sacroiliitis which is due to inflammation of the sacroiliac joint and is considered to be the most common symptom of AS. The pain stretches from the lower part of the back to the buttock, but rarely occurs below the knees. The best way to diagnose and to grade the sacroiliitis is by radiography or magnetic resonance imaging (MRI).

Synovitis is a common symptom in rheumatoid disease, which implies inflammation in joints. The joints that commonly are affected in patients with AS are knees, hips and ankles while upper limb joints and shoulders rarely are affected. The inflammation can lead to deformity and problems to move.
Inflammatory bowel disease (IBD) is more common in people with AS than in healthy people and both Crohn’s disease and ulcerative colitis are more frequent; 6-25% of patients diagnosed with AS. Around 60% of AS patients are believed to have some sort of clinical changes in the bowel.

AS patients have a higher risk of developing osteoporosis. The spine is the area that is most commonly affected with fractures accruing in the vertebrae. The risk of osteoporosis depends on the severity of the AS. When a fracture in the vertebra occurs, the vertebra collapses. If the fractured vertebra is not treated, the back will get crooked after a few fractions, because the healing makes it wedge-shaped. Symptom of a fracture in the spine is back pain in the region [1].

It is also common that the eyes are affected in AS patients; they can develop iritis which is an acute anterior uveitis. It is a critical condition and the patients need treatment to avoid impact of visual field and scarring of the pupil. Blurring of vision, sore and red eyes can be symptoms of iritis and affects approximately one in three patients.

Psoriasis is also a common disease affecting up to 25% of patients with AS. It has also been shown that pulmonary diseases are more common in these patients. In a recent study from Norway, it was shown that 18% of AS patients showed a restrictive pattern when spirometry was preformed, compared to the control group and reference data [2].

AS is difficult to diagnose because many symptoms are general and due to that it takes long time to discover and diagnose AS. Contrary to other rheumatic diseases, AS is sero negative for rheumatoid factor, that makes diagnoses difficult because rheumatoid factor is important for diagnosis of rheumatoid diseases [3].

For diagnosis of AS, the modified New York criteria for ankylosing spondylitis is usually used. To be diagnosed with AS, the patient need to have at least one of criteria 1-3 and have
criteria 4; the first is pain in the lower part of the back for at least 3 months that will improve with exercise. The pain should not get better with resting. The second is having problem with movement of the lumbar spine. Decreased expansion of the chest is the third criteria. The fourth and last is sacroiliitis; unilateral grade 3-4 or bilateral grade 2-4.

If the patients does not fulfill the criteria for AS, the Amor’s classification criteria for spondyloarthritis can be used to see if the patient has a risk for develop some sort of SpA. In addition to these criteria, presence of a HLA-B27 gene is considered as a risk factor. Another risk factor for developing AS is male gender. The disease is more common in men than women, with 65-80% of patient with AS patients being male [6].

Today the exact cause of AS is unknown. It is believed that multiple factors in combination will lead to AS. The strongest factor known to have an impact is the HLA-B27 gene, 90% of all AS patients have this genotype. In twin studies, it has been shown that HLA-B27 alone contributes to around 30% of the risk of developing AS. The HLA-B27 gene is also present in 50-57% in other SpA diseases, 5% of all carriers of HLA-B27 are at risk to develop some form of SpA disease. People that are homozygotes for the HLA-B27 gene have higher risk of developing AS than those that have HLA-B27 heterozygosity. Furthermore, people that are HLA-B27 positive develop AS at a younger age than those that are HLA-B27 negative. How HLA-B27 actually contributes to AS is unknown, but there are many theories. It has been observed that some people express more HLA-B27 protein in antigen-presenting cells than normal. Also misfolding of the HLA-B27 protein presented on the cell surface has been shown. HLA-B27 is the only HLA gene type that has been described misfolded. The reason for the misfolding is unknown but one theory is that endoplasmatic reticulum (ER) stress may be a contributing factor [1].
Another theory is that bacteria is a trigging factor for development of AS. It has been shown that 70% of patients with AS have IgG-antibodies that recognize a 30-KDa band from *Salmonella*. AS patients in general have a higher antibody level of IgG and IgA to an antigen from *Salmonella* [5]. Other bacteria that might be involved are *Chlamydia, Campylobacter, Yersinia and Shigella*, all described associated with other SpA diseases. All bacteria that have been associated with AS are gram-negative [4].

There is no cure for AS today, but it is possible to alleviate the symptoms. The most important treatment for patients is exercise/physical therapy. It has been shown that 50min of exercise 3 times a week will decrease the stiffness and ease the back pain [6]. An additional treatment to ease the pain and the inflammation is to use non-steroidal anti-inflammatory drugs (NSAIDs). It is usually the first line treatment for AS. If the patient is allergic or cannot take NSAID for other reasons, disease modifying anti-rheumatic drugs (DMARD) can be used. However, compared to other rheumatic diseases, this option is not as efficient for treatment of AS and other SpA diseases. Glucocorticoids can also be used to ease the pain and morning stiffness, but only for short-term treatment due to side effects. Anti-tumor necrosis factor (TNF) or TNF blockers is another treatment that can be used to relive the symptoms; it is an anti-inflammatory medication that inhibits the effect of TNF-α [7].

The HLA genes are synonymous to major histocompatibility complex (MHC). The MHC is the general name for the genes in all species, called HLA in humans. The HLA genes are involved in the immune system, which can cause autoimmune reactions if something goes wrong in the transcripts of the gene [8].

There are two different types of HLA genes, class-I and class-II. The proteins for class-I are present on the surface of all cells in the body, and present fragments of intracellular antigen
for T-cell recognition of infection or other foreign fragments in the cell. The class-II molecules are expressed on B-cells, macrophages and dendritic cells. Instead of showing antigens from within the cell, the class-II molecules present antigens picked up from the environment. The HLA genes are made up by three different loci, for class-I named A, B and C and while class-II loci are called DP, DQ and DR. Each of these loci has one α- and one β-chain. The α-chain is also called heavy chain and the β-chain is called β2-microglobulin (β2M) or short chain. The α-chain has three domains α1, α2 and α3 equivalent to the A, B, and C loci. The β2M has only one domain and is not connected to the cell surface in the same way as the α-chain. The β2M attaches to the α-chain with covalent bonds. The α1 and α2 domains create a space where the peptide fragment is presented [8].

The B locus, allele 27 in the HLA class-I gene, is the most important B locus for the risk of developing AS. The B loci have different alleles with different subtypes of each allele [8]. Of the subtypes of HLA-B27, some are more frequent in AS while others are not associated with AS at all. The HLA-B*2702, HLA-B*2704 and HLA-B*2705 are most common in AS, but other subtypes have also been seen. HLA-B*2706 and HLA-B*2709 are not associated with AS. The difference between subtypes that are associated with AS and those that are not are only one or two amino acids [1].

When the HLA-B27 is misfolded it is usually the α-chain that misfolds in the ER. The misfolding of the protein in ER could lead to ER-stress and the unfolded protein response will start. When the unfolded protein response gets activated, macrophages will start to produce cytokines, which will lead to an inflammatory reaction. This seem to be the case in AS, which is characterized by repeated inflammation in the joints leading to deformation of the joints. A possible reason why HLA-B*2706 and HLA-B*2709 is not associated with AS could be that they fold more efficiently than other HLA-B27 subtypes [1].
AS and SpA is common in Ireland which lead to a request from the immunology department of St. James hospital in Dublin, Ireland to develop a PCR based method. A good detection method for these diagnoses would save time as diagnosis would be faster and it will be easier to diagnose the patients. Therefore, the aim for the project was to detect the HLA-B27 gene by using PCR. We decided to use the protocol from a previously published article describing a PCR for detecting HLA-B27 [9].

**METHODS**

**SAMPLES**

The samples were provided by the rheumatology department at St. James hospital, Dublin Ireland. Twenty-two patient samples were included, 17 with AS diagnosis and five suspected to have AS. Whole blood in EDTA tubes was donated. One of the patients had previously had the HLA genes sequenced and was known to have the HLA-B27 gene.

**DNA EXTRACTION**

DNA extraction was based on a method that was used at the university, but was optimized for use of frozen EDTA samples and for each sample a double extraction was performed. 500µl of whole blood was added to the tube and 900µl of red blood cell lysis solution (155mM ammonium chloride, 10mM Potassium hydrogen carbonate, 1mM EDTA) was added. The tube was incubated for 10min in room temperature. The tube was mixed by inversion. The
result was a transparent red solution. After incubation, the tube was centrifuged at 20 800 x g for 1 min which created a white pellet. The supernatant was removed, about 20µl was left in the tube that was re-suspended with the pellet and the lysis step was repeated with 500µl of red blood cell lysis solution and the tube was incubated for 5 min. The tube was mixed by inversion occasionally and after incubation it was centrifuged as above for 1 min. The supernatant was then removed and the pellet was re-suspended by vortexing. Then 300µl of white cell lysis solution (25mM EDTA, 2% SDS) was added. The solution was mixed by pipetting to re-suspend the pellet, and the mixture was thereafter incubated at room temperature for 2 min. 100µl protein precipitation solution (10mM ammonium acetate) was added and the mixture was vortexed for 10 sek. Then the tube was centrifuged for 1 min at 20 800 x g. Isopropanol (300µl) was added to new tubes to which 300µl of the supernatant from the extraction was added. The solution was mixed by inversion of the tube. The tube was centrifuged for 5 min at 20 800 x g and the supernatant was removed. 300µl of 70% ethanol was added to precipitate the DNA. The tube was vortexed and centrifuged as above for 5 min and the supernatant was removed. The precipitate was left to dry on paper tissue after which 25µl of TE buffer (10mM Tris.Cl pH 8.0, 1mM EDTA) was added and the tube was incubated at 65°C for 10 min.

After DNA extraction, the concentration and purity of the DNA was determined using nanodrop (nanodrop 2000, Thermo, USA). The DNA was then stored in the freezer at -20°C.

PCR

Primers
The primers that was used in this project for the PCR was for the β-globin; forward, GH20 – GAA GAG CCA AGG ACA GGT AC and reverse, PC04 – CAA CTT CAT CCA CGT TCA CC. For HLA-B7 forward – GGG TCT CAC ACC CTC CAG AGC, HLA-B27 forward, E91s – GGG TCT CAC ACC CTC CAG AAT and the reverse primer was universal for all HLA-B genes HLA-B reverse, e136as – CGG CGG TCC AGG AGC T.

Beta-globin PCR

β-globin PCR was used as a control in the PCR to verify that the PCR had worked. The master mix for the β-globin was made using 1µl Primer mix (100ng/µl), 0.5µl dNTP stock (10mM), 2.5µl 10xPCR buffer (10xPCR buffer, Invitrogen), 0.2µl Taq (Taq DNA polymerase, Invitrogen) and 16.8µl milliQ water for each PCR. The total volume of master mix added in to each PCR tube was 21µl. Of DNA or milliQ water (blank) 1µl was added to the PCR tube. Then MgCl$_2$ (50mM MgCl$_2$, Invitrogen) and milliQ water was added, the total volume of MgCl$_2$ and water was 3 µl. The individual volume depended on the MgCl$_2$ concentration. A MgCl$_2$ titration was performed using 0.5, 1, 1.5, 2, 2.5, and 3mM concentration of MgCl$_2$. The total volume in each tube was 25µl.

The PCR started with denaturation for 2min at 94°C. Thereafter 35 cycles with a denaturing step at 94°C for 30sek, annealing at 55°C for 30sek and elongation at 72°C for 30sek was carried out. The PCR ended with an elongation step at 72°C for 5min.

To determine the PCR results, the PCR product was separated on a 1% agarose gel in TBE, that was stained with 2µl of Gel red (Gel red nucleic acid stain, Biotium, lot; 10G0428). The Gel red was added before casting the gel. 2µl of loading buffer and 10µl of PCR product was
mixed and added in to the well. The gel was run for 45min at 100V and then visualized with a UV camera.

HLA-B7 plasmid PCR

HLA-B7 gene was used as control for the PCR. The sequence had been synthesized in a plasmid (Eurofins MWG operone) and the PCR was performed using the same conditions as described before for the β-globin PCR. Primers for the HLA-B7 gene were added in with the plasmid DNA, 1µl (1ng/µl) plasmid was used and 1µl (1µmol/µl) primer. MgCl₂ titration was carried out to determent the optimal concentration. A tube with β-globin primers and human DNA was included as a control to ascertain that the PCR step was working. In one of the runs, a tube with HLA-B27 primers was used instead of the HLA-B7 primers, to see if the HLA-B27 primers amplified the HLA-B7 gene when the MgCl₂ concentration was 1.5mM.

HLA-B27 PCR

The PCR for HLA-B27 was performed using the same conditions as for the HLA-B7 and β-globin PCR. To determine which patients that were HLA-B27 positive, 2 different patient samples were run in a PCR with “platinum blue PCR supermix” (platinum blue PCR supermix, invitrogen). The patient DNA that had a clear band at expected size was used as template. Later in the project, this patient was confirmed to be HLA-B27 positive. The amount of primers that was added was 1µl (1µmol/µl) and 1µl of DNA was used, the DNA had a concentration between 60-250ng/µl.

MgCl₂ titration was carried out to find the optimal MgCl₂ concentration. The volume of Taq, dNTP, 10xPCR buffer, DNA and primers was the same as for the β-globin PCR, but the
milliQ water was reduced to 14.8µl and the total volume of MgCl₂ and water was increased to 5µl, while the primers that were used was the same as for HLA-B27. A tube with β-globin gene was always added in the PCR as a control. When the optimal MgCl₂ was determined, new PCR was performed to find the optimal annealing temperature. The tested annealing temperatures were 55°C, 58°C, 60°C and 62°C. A full MgCl₂-titration was performed at each annealing temperature to see if the optimal MgCl₂ concentration changed with annealing temperature.

A multiplex PCR was carried out where the β-globin primers were added in the same tubes as the HLA-B27 primers. The multiplex PCR was preformed with the same solutions as above for the β-globin PCR and with the “platinum blue PCR supermix” solution. The PCR was first run with 35 cycles and then repeated with 40 cycles to increase the amount of product. The “platinum blue PCR supermix” contains a hot-start Taq that is connected to a monoclonal antibody that inhibits start of Taq synthesis in advance. The antibody is thermo-labile, when heated it is removed from the Taq which will then become active. By using a hot-start Taq, the risk of getting primer-dimers is reduced.

A gradient multiplex PCR with 40 cycles was run with the “platinum blue supermix”. The temperatures that were tested were 56°C, 58°C, 60°C, 62°C and 64°C. A gradient multiplex was also performed with a HLA-B27 positive sample and the HLA-B7 primers to investigate the best temperature for specific amplification of the HLA-B27 gene.

After finding the optimal temperature for the multiplex PCR, all patient samples were tested in a multiplex PCR with HLA-B27 and β-globin primers. The “platinum blue supermix” was used and the annealing temperature was 58°C, with 40 cycles. Patient sample 1-12 were tested in both regular and multiplex PCR, with the HLA-B7 and the β-globin primers as controls while sample 13-22 were analyzed in multiplex PCR. Ten of patient
samples were used in a blinded test to see if the results were the same as before. It tested if the analyze was reproducible.

For visualizing of the PCR products a 1.5% agarose gel was performed.

The optimal PCR for detecting HLA-B27

23µl of the “platinum blue supermix” was used. The primer concentration was 1µl (1µmol/µl) in total, 0.5µl of the HLA-B27 primers and 0.5µl of the β-globin primers. 1µl (60-250ng/µl) of DNA was also added to the mix giving a total volume of 25µl.

The PCR program started with a denaturation step for 2min at 94°C and continued with 40 cycles with a denaturation step for 30sek at 94°C, an annealing step for 30sek at 58°C and an elongation step for 30sek at 72°C. The PCR ended with an elongation step at 72°C for 5min.

After PCR, 10µl of the PCR product was mixed with 2µl of loading buffer and added to the gel, which was run as described earlier.

RESULTS

DNA extraction

The quantity of DNA after extraction varied considerably, as judged from the nanodrop results. The DNA that was used in the PCR had a concentration between 60ng/µl to 250ng/µl and the median was 125ng/µl. The median purity (260/280) of the DNA was 1.85 (1.80-2.00).
PCR

β-globin

The β-globin gene amplification did not vary with MgCl$_2$ concentration (figure 1). The MgCl$_2$ used was 1.5mM when β-globin was used as a control.

![PCR products after amplification of β-globin PCR and an MgCl$_2$ titration. 1= DNA ladder, 2= negative control, 3= 0.5mM MgCl$_2$, 4= 1mM MgCl$_2$, 5= 1.5mM MgCl$_2$, 6= 2mM MgCl$_2$, 7= 2.5mM MgCl$_2$, 8= 3mM MgCl$_2$.](image)

Plasmid HLA-B7

The optimal MgCl$_2$ was found to be 1.5mM in the PCR but the concentration was not very critical in the range 1mM to 3.0mM MgCl$_2$ (figure 2).

The HLA-B27 primers did not amplify HLA-B7 gene which shows that the HLA-B27 primers are specific for the HLA-B27 gene (figure 2, lane 9).
Figur 2, Amplification of HLA-B7 plasmid DNA and MgCl₂ titration. The HLA-B27 primers did not amplify the HLA-B7 gene. 1= DNA ladder, 2= negative control, 3= 0.5mM MgCl₂, 4= 1mM MgCl₂, 5= 1.5mM MgCl₂, 6= 2mM MgCl₂, 7= 2.5mM MgCl₂, 8= 3mM MgCl₂, 9= Plasmid DNA + HLA-B27 primers 1.5mM MgCl₂, 10= β-globin.

HLA-B27

In the test with the “platinum blue supermix” to find a HLA-B27 positive sample, one of the two samples was identified positive. The same sample had also been confirmed positive previously at the hospital (figure 3).

Figure 3, PCR amplification of HLA-B27. Two samples, one is HLA-B27 positive, 1= DNA ladder, 2= negative control, 3= Positive sample, 4= Negative sample, 5= β-globin.

The optimal MgCl₂ concentration for the HLA-B27 PCR was determined to 1.5mM but concentrations between 1-3mM worked.
Multiplex PCR

Multiplex PCR for HLA-B27 and β-globin was carried out using two different mix solutions. The multiplex with the “platinum blue supermix” (figure 4) gave a more intense band than the standard solutions.

![Image of Multiplex PCR amplification of HLA-B27 and β-globin genes using “platinum blue supermix”](image)

Figure 4, Multiplex PCR amplification of HLA-B27 and β-globin genes using “platinum blue supermix”, 1= DNA ladder, 2= negative control, 3= HLA-B27 + β-globin primers, 4= β-globin primers.

Gradient PCR

A gradient multiplex PCR was carried out. The optimal temperature was 58°C. PCR worked in temperatures between 56°C-62°C.

![Image of Gradient Multiplex PCR amplification of HLA-B27 and β-globin genes](image)

Figure 5, Gradient Multiplex PCR amplification of HLA-B27 and β-globin genes HLA-B27 primers + β-globin primers, 1= DNA ladder, 2= negative control, 3= 56°C, 4= 58°C, 5= 60°C, 6= 62°C, 7= 64°C.
The gradient PCR with the HLA-B7 primers and the HLA-B27 positive sample showed that the PCR worked in temperatures between 56°C-62°C and that the optimal temperature was 58°C (figure 6).

![Figure 6, Gradient Multiplex PCR amplification with β-globin primers + HLA-B7 primers with β-globin and HLA-B27 positive DNA to see that the HLA-B7 primers did not amplified the HLA-B27 positive DNA. 1= DNA ladder, 2= negative control, 3= 56°C, 4= 58°C, 5= 60°C, 6= 62°C, 7= 64°C.]

In the test where all patients’ samples were tested, it was shown that 16 patients were HLA-B27 positive and 6 patients was HLA-B27 negative. This means that 72.7% of the patients were HLA-B27 positive. In the test with patient samples 1-12 where they were also analyzed using the B7 primers, no non-specific amplifications were shown.

Assay repeatable

In at blind test, 10 out of 10 samples were correctly analyzed and the sensitivity of the PCR was subsequently determined to 100%. It concludes that the assay are repeatable.

**DISCUSSION**
It is difficult to diagnose AS and the causes of AS is unknown, but one of the most influential factors is the HLA-B27 gene. To detect the gene would improve the diagnosis of the disease.

PCR is a method which is relatively easy and fast as the results can usually be presented the same day and it is therefore an often used method in hospital based laboratories.

A PCR method for HLA-B27 gene has previously been published and was used as a starting point in the project. Our optimization using our PCR machine resulted in a slightly different protocol; additional cycles were needed, the primer concentration was lower and the optimal annealing temperature was higher. One reason why the results differed is that when primer concentration is lower, more cycles and higher annealing temperature are needed. The lower primer concentration was important to get rid of the primer-dimers.

The project was initiated with optimization of the DNA extraction conditions. The standard protocol that was used was designed for fresh whole blood, not frozen. The first problem was to get a good yield of DNA in every extraction. Freezing of the blood results in decreased yield of DNA compared to fresh blood [10]. To get more DNA in every extraction the starting blood volume was increased and the lysis step of the red blood cells was repeated to get better purity. It is not known why the lysis steps needed to be repeated as the red blood become lysed when frozen. Another factor that could affect the DNA concentration is the number of leucocytes in the blood. The DNA concentration varied between different patients and was generally lower in the blood from healthy donors. This could be due to the fact that patients that have an autoimmune disease have increased concentration of leucocytes which results in more DNA.

The primer concentration was reduced in our study compared to previous published data [9]. Previously, 5µmol/µl of primers was used in each reaction, which in this study was found to create primer-dimers. Reduced primer concentration to 1µmol/µl removed the presence of
primer-dimers. In another article about HLA-B27 PCR, the concentration of the primers was 0.7µmol/L [11]. This is more similar to the concentrations of primers used in our study which shows that a lower primer concentration works well.

In the HLA-B7 PCR, the MgCl₂ concentration was important. The MgCl₂ concentration should be higher than 0.5mM and lower than 3mM. The optimal concentration was 1.5mM.

When multiplex PCR with HLA-B27 and β-globin was preformed, it was important to optimize the conditions to get equal amount of each primer. However the numbers of copies initially became too low. Hot-start Taq resulted in less unspecific bindings and primer-dimers. To increase the number of copies in these PCRs, the number of cycles was increased from 35 cycles to 40 cycles, which gave more product and thereby getting better results.

Another interesting discovery with the multiplex PCR was that the annealing temperature needed to be optimized. Higher annealing temperature, 64°C, resulted in HLA-B7 unspecific synthesis of HLA-B27 products. The β-globin product also became weaker in intensity with higher annealing temperature. Therefore, an optimized annealing temperature was important to avoid false positive bands which could lead to misdiagnosis of patients. For the β-globin PCR the optimal annealing temperature are 55°C, that is also described by Wu D.Y et.al. where they also had a β-globin PCR with 55°C annealing [12].

The importance of having the β-globin as control was obvious after analysis of all patient samples. In one if the PCR-tubes the reaction did not work, there were no amplification of neather β-globin nor HLA-B27. Without control, the sample would have been interpreted as negative for HLA-B27.

For the PCR to be approved for diagnosis, more evaluation is needed. In this study, only 22 patient samples were included and only one of the samples was known to be HLA-B27
positive from previous analysis. To evaluate this PCR method, the product could be sequenced to ensure correct results. Another alternative could be to include more samples where it is known which samples are positive or negative from previous analysis. In previous studies using PCR to detect HLA-B27 61, 270 and 371 samples, known to be HLA-B27 positive were included [9, 11, 13]. This suggests that more known positive samples is needed to determine the specificity/sensitivity of this assay.

The best condition for detection of the HLA-B27 gene with PCR in this project was to use the “platinum blue supermix” to avoid unspecific binding. The MgCl$_2$ concentration in the mix should be 1.5mM.

This PCR method could be developed for real-time PCR. This would save both time and money because the last electrophoresis step is not included in the analysis. Today a majority of diagnostic tests for HLA-B27 uses real-time PCR and this PCR could be used as a starting point to develop a real-time PCR for the hospital. Developing a PCR is cheaper than buying pre-made kit [13, 14].

In summary; the developed PCR method could be used to detect the HLA-B27 gene and be a starting point for development of a real-time PCR.

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REFERENCES


